

Positive Role of Nodulation on the Establishment of *Rhizobium japonicum* in Subsequent Crops of Soybean

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Abstract. The influence of soybean nodulation on the establishment of *Rhizobium japonicum* in *Rhizobium*-free soil was examined. Seeds of nodulating (Rj_1) and nonnodulating (rj_1) isolines of soybeans and four other crop species (cowpeas, mungbeans, corn, and alfalfa) were grown in field plots that were inoculated with a genetically marked strain of *Rhizobium* (strain I-110 ARS) and the following year nodulating soybeans were grown in these plots and were inoculated with a different genetically marked subline of the same strain (strain I-110 FN). The proportion of nodules containing strain I-110 ARS relative to strain I-110 FN was determined and interpreted as reflecting the relative numbers of the two genetically marked sublines in the soil. The results clearly demonstrate that nodulation with the specific host plant (soybeans) has a significant positive role in the establishment of *Rhizobium* in *Rhizobium*-free soil and suggests that alfalfa plants diminish the establishment of soybean rhizobia in soil.

Research on the dynamic interactions of *Rhizobium* bacteria and their specific host plants has been reviewed [5]. Yield responses of soybeans to applied inoculants of *Rhizobium* are generally found only in fields not previously planted in soybeans [1]. The failure of new inoculum strains placed in populated soils to effect increased yields is a perplexing problem. Fundamental knowledge of the ecology of the legume/*Rhizobium* symbiosis is needed in order to increase crop yields through the selective breeding or genetic engineering of *Rhizobium* for improved biological nitrogen fixation. In this investigation, we employed genetically marked strains of *Rhizobium japonicum* to determine the role of nodule formation and the effect of crop species on the establishment of *R. japonicum* in *Rhizobium*-free soil. It is hoped that the concepts developed will be applicable to the more complex situation of *Rhizobium*-populated soybean production fields.

Materials and Methods

Bacterial strains. *Rhizobium japonicum* strain I-110 ARS was derived from strain I-110 [4] following sequential selection of genetic markers for resistance to azide (10 μ g/ml), rifampin (500 μ g/ml), and streptomycin (1 mg/ml). Strain I-110 FN has genetic markers for resistance to 10 μ g/ml 5-fluorouracil and 500 μ g/ml nalidixic acid, and was also isolated from strain I-110 following

sequential isolation and purification of spontaneous antibiotic-resistant mutants occurring at frequencies of about 10^{-7} to 10^{-8} .

Field experimentation. Plots 1.8 m \times 1.2 m with 0.9 m grass interspaces between plots were established on a Donlon fine sandy loam soil at the University of Maryland Tobacco Farm at Upper Marlboro, Maryland, on 30 June 1978. The soil was essentially free of *R. japonicum* as determined by plant tests in the greenhouse. A randomized block design was used with four replications. Seed of "Clark" soybeans and the nonnodulating isolate, Clark rj_1rj_1 [8], were derived from stocks obtained from the soybean Genetic Type Collection courtesy of R. L. Bernard, USDA, University of Illinois, Urbana, Illinois. Other seeds used were "Burken" mungbeans (*Vigna radiata* [L.] Walp.), "Funks G4646" corn (*Zea mays* L.), and "Arc" alfalfa (*Medicago sativa* L.). Seed of Arc alfalfa was broadcast seeded and the other crops were planted in rows 0.3 m apart. Three 1.8 m rows were inoculated at seeding with about one liter of broth culture containing about 10^8 viable cells per ml; alfalfa plots also received one liter of inoculum.

Mungbeans and cowpeas were harvested for seed in the fall. Plant materials in the other plots were returned to the soil. In 1979, all plots were planted with Clark soybeans and inoculated with strain I-110 subline 5-*fu*-1 nal-23 (I-110 FN) carrying 10 μ g/ml 5-fluorouracil resistance and 500 μ g/ml nalidixic acid resistance, in the same manner as strain I-110 ARS had been introduced the previous year.

Strain identification. At 47 days after planting, 60 nodules (10 nodules from each of 6 plants) from each of the four replicate plots of each treatment were analyzed for strain identity. Nodules were surface-sterilized in 3% H_2O_2 for 1 h and then rinsed

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three times with sterile distilled water. Nodules were then squashed and aliquots were plated onto selective media containing either 500 µg/ml nalidixic acid (N500), or 500 µg/ml rifampin sv and 1 mg/ml streptomycin (R500 + S1000) (all from Sigma Chemical Co., St. Louis, Missouri). Scoring of patches for growth or absence of growth was done after 5 to 7 days incubation at 30°C for R500 + S1000 medium and after 10 to 14 days for N500 medium.

Results

The influence of the nodulating soybean on the establishment of *Rhizobium japonicum* strain I-110 ARS in *Rhizobium*-free soil was examined and compared with the influence of nonnodulating (rj_1) soybeans, mungbeans, cowpeas, corn, and alfalfa. The influence that the soybean genotype and crop species had on the establishment of the strain in the first year was measured in the second year as follows: (i) A different genetically marked subline of strain I-110, I-110 FN, which has five dissimilar markers, was used as an inoculum in the second year of the experiment when all the plots were planted with nodulating (Clark) soybeans. (ii) The proportion of nodules containing strain I-110 ARS relative to strain I-110 FN was determined and interpreted as reflecting the relative numbers of the two genetically marked sublines in the soil at the time of infection due to a differential in persistence of strain I-110 ARS from the first year.

This interpretation is possible because these sublines of *R. japonicum* strain I-100 would be expected to have equal competitiveness by virtue of their derivation from the same source. Furthermore, the use of a single host genotype, Clark soybeans, in the measurement procedure in the second year would obviate the effect of any differences in strain competitiveness on treatment differences. Plant selection and differential responses to environmental factors are therefore excluded from affecting the relative proportion of infection by either genetically-marked subline.

Nodules formed by strain I-110 ARS contained bacteria capable of growth on a selective medium containing rifampin or streptomycin, whereas nodules formed by strain I-110 FN contained bacteria capable of growth on a selective medium containing nalidixic acid, but not on rifampin or streptomycin. The relative proportion of nodules containing strain I-110 ARS was significantly higher in plots where nodulating soybeans had been grown when compared with plots where nonnodulating rj_1 soybean, mungbeans, cowpeas, corn, or alfalfa had been grown (Table 1). The results also indicated that the

persistence of *R. japonicum* strain I-110 ARS population was not, in comparison, significantly different among corn, nonnodulating rj_1 soybeans, mungbeans, or cowpeas. In comparison with either nodulating or nonnodulating rj_1 soybeans, a significantly lower proportion of nodules containing strain I-110 ARS was found in plots where alfalfa had been grown and inoculated with this strain the previous year.

Table 1. The influence of soybean nodulation genotype and crop species on the establishment of *Rhizobium japonicum* strain I-110 ARS in soil: relative frequency of strain I-110 ARS in nodules of soybeans inoculated with strain I-110 FN.^a

First year soybean genotype and/or crop species	Mean proportion (%) strain I-110 ARS
Clark (Rj_1Rj_1) soybeans	53.4 A ^b
Clark (rj_1rj_1) soybeans	30.6 B
Mungbeans	25.4 BC
Cowpeas	23.7 BC
Corn	13.0 BC
Alfalfa	6.7 C

^a Strain I-110 ARS (see Materials and Methods) is carried over from the first year whereas strain I-110 FN is the inoculum strain in the second year. Thus, the established population is challenged by a different genetically marked subline.

^b Means followed by the same letter are not significantly different at the 0.05 probability level as tested by Duncan's Multiple Range Test.

Discussion

Nodulation of the specific plant host by the *Rhizobium* microsymbiont has long been generally assumed to positively benefit *Rhizobium* bacteria. Reyes and Schmidt [6] compared the populations of *Rhizobium japonicum* strain 123 in the rhizosphere of corn, wheat, and nodulating and nonnodulating isolines of soybean and found no evidence for a specific stimulation of bacterial proliferation by the host plant. The effect of nodulation per se was disregarded because their purpose was to determine whether specific rhizosphere stimulation occurs prior to or concurrent with the infection process and nodulation. In this study, we designed an experiment to test the role of nodule formation and the effect of crop species on the establishment of *R. japonicum* in *Rhizobium*-free soil.

Our results show that nodulation of the soybean promotes establishment of *R. japonicum* in soil. The Clark and Clark rj_1 isolines are genetically identical except for the rj_1 locus and genetic material very closely linked to the rj_1 locus. The different influence on rhizobial establishment shown by the

two lines is due to the Rj_1 allele determining the presence of nodules on the roots [2]. Research by Clark [2] and Elkan [3] has shown no negative effect of the rj_1 allele on the proliferation of rhizobia in the rhizosphere. In their studies the numbers of rhizobia found on the roots rj_1rj_1 plants were at least as high as those found on Rj_1Rj_1 plants. Our study conclusively demonstrates that nodule formation as controlled by the Rj_1 gene has a clear positive role in establishing *Rhizobium* populations in *Rhizobium*-free soil. We concur with others [6] that increases of *Rhizobium* populations in soil probably result from the release of viable bacteria from senescent nodules.

Because the methods of inoculation used for the alfalfa plots and the soybean plots in the first year were dissimilar, the differences in the establishment of strain I-110 ARS may be due either to the method of inoculation or to a negative effect of alfalfa on the establishment and persistence of the soybean *Rhizobium* in the soil. A negative effect of alfalfa would not be surprising in the light of the work by Thorne and Brown [7] who showed that the unheated juices extracted from legumes such as soybeans were bactericidal for nonhomologous microsymbionts such as *Rhizobium trifolii* presumably due to the production of specific bactericidal proteins. Our observation that alfalfa plants may diminish the establishment of soybean *Rhizobium* could be accounted for by the production of proteins that are bactericidal for *R. japonicum*. In the evolution of symbiosis, such a phenomenon would serve to prevent infection and nodulation by genetically related but incompatible (non-nitrogen-fixing) strains. Further research in this area is warranted because likely future developments could include the control of populations of relatively inefficient *Rhizobium* strains in soils so that yield responses can be obtained from inoculation with *Rhizobium* strains that fix more nitrogen.

Our conclusions might be summarized as follows: (i) Comparison of the results from plots

containing nodulating soybeans with those containing the nonnodulating (rj_1rj_1) isoline clearly establishes that nodulation per se can result in a biological advantage for the microsymbiont. Genetically marked strains of *Rhizobium japonicum* were useful for demonstrating a significant positive role of the host plant nodulation in the establishment of *Rhizobium* in *Rhizobium*-free soil. (ii) Comparison of the results from plots containing either nonnodulating soybeans, mungbeans, cowpeas, or corn shows no apparent significant differences in the effect of these crops on the establishment of *Rhizobium* in soil. Comparison of the above mentioned results with those obtained with alfalfa suggests that alfalfa may diminish the establishment of *R. japonicum* in the soil, perhaps by producing proteins that are bactericidal for *R. japonicum*.

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